2178-Plat
Activity of Both PKA and Camkii is Required for Maximal RyR Sensitivity Under Beta-Adrenergic Stimulation
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In cardiac myocytes, calcium sparks exhibit time-dependent refractoriness such that triggering of a second spark soon after an initial spark has terminated is improbable. Recent studies in rat ventricular myocytes suggested that spark amplitude recovery is controlled by local sarcoplasmic reticulum (SR) refilling whereas refractoriness of spark triggering depends on both refilling and ryanodine receptor (RyR) sensitivity.
Here we examined spark refractoriness in mouse ventricular myocytes by exposing Fluo-3 loaded quiescent cells to 50 nM ryanodine, recording sparks with a confocal microscope, and analyzing the repeated sparks that were produced at singular RyR clusters.
Beta-adrenergic stimulation accelerated spark amplitude recovery and decreased median spark-to-spark delay compared to control. Spark amplitude recovery was also accelerated/decelerated, respectively, by either activating or inhibiting PKA with forskolin or H89. Spark-to-spark delays were not affected by forskolin but increased in H89. Experiments performed on S2808A mice with a mutated PKA phosphorylation site corroborate these results.
Forskolin results suggest that during beta-adrenergic stimulation pathways other than PKA can be activated. Beta-adrenergic stimulation in the presence of PKA or CaMKII blockers (H89 and KN93, respectively) were studied. In both conditions, time constant of amplitude recovery was increased and spark-to-spark delay was slightly higher than control. Employing a mathematical model allowed us to gain further insight into experimental results. Under control conditions SR refilling is enhanced due to activation of SERCA via endogenous PKA activity. Additional activation of either PKA or CaMKII is sufficient to accelerate spark amplitude restitution through faster refilling. However, inhibition of either pathway prevents RyR sensitivity to be increased. Thus, activity of both kinases is necessary to explain the changes in RyR gating observed during beta-adrenergic stimulation.

2179-Plat
Critical Requirements for the Initiation of a Cardiac Arrhythmia in Heart: Cell Number
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Many features of an intact functioning heart provide a margin of safety with respect to the generation of cardiac arrhythmias. We have been investigating Ca^{2+} dependent arrhythmias and the requirements for such pathological activity including features that focus on the margin of safety. One of the primary issues is the number of cells involved in the initiation process. If too few cells are needed to initiate the arrhythmia, arrhythmias will be rampant. If too many cells are needed, then it is difficult to explain how known arrhythmias occur. Thus the balance is one of the critical parameter to be estimated to validate the model and also to develop a useful set of computational tools to examine lethal cardiac arrhythmias. It has been suggested in published work that, a large number (~800,000) of ventricular myocytes be depolarized to overcome the electrotonic load of the heart. In our recent work we have re-examined this question in light of a revised stochastic model of electrical and Ca^{2+} activity. We have found that as few as 12 myocytes may be needed to initiate an ectopic cardiac arrhythmia. This reduction of over 4 orders of magnitude reflects important improvements in the modeling. These changes include the stochasticity of the Ca^{2+} modeling, improved assumptions regarding the ionic currents in heart cells and updated geometries. We will present and discuss these critical changes and the justification for them. Our new findings regarding the initiation of spontaneous arrhythmias are in line with functional data from the heart. This new finding suggests a mechanism by which the generation of arrhythmias is possible while still maintaining a high safety margin.

2180-Plat
Structural and Functional Alteration of RyR Clusters After Remodeling in Persistent Atrial Fibrillation
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In chronic atrial fibrillation (AF), abnormalities in Ca^{2+} release from RyR have been implicated as major factors contributing to arrhythmia and contractile dysfunction, but the relation to RyR organization remains unknown. Using STED microscopy we examined RyR cluster morphology in isolated atrial myocytes from sheep with persistent AF (N=6, 16-23 weeks of AF) and age matched control (Ctrl) animals; in parallel experiments we measured Ca^{2+} sparks in permeabilized myocytes. STED measurements revealed RyR clusters typically contained 15 contiguous RyR and on average this did not differ between AF and Ctrl. However, the nearest neighbor distance between clusters was reduced in AF. Grouping of clusters within 150 nm as functional Ca^{2+} release units (CRU) indicated that in AF these units exhibit increased fragmentation, with more clusters per functional unit. Measurement of Ca^{2+} sparks in permeabilized myocytes revealed a >50% increase in spark frequency and a higher prevalence of macrosparks. Spark time-to-peak (TTP) and duration were also increased, but width was reduced. Measurement of the intrinsic cellular buffer capacity showed this was reduced in AF. Using computational modeling it was found that the increased TTP and duration can be ascribed to the increased number of clusters per functional grouping in AF. The observed increased CRU fragmentation and reduction in Ca^{2+} buffering, can increased occurrence of sparks and macrosparks in AF. In conclusion, ultrastructural reorganization of RyR clusters within the functional units contributes to overactive Ca^{2+} release with increased chance of propagating events in AF.

2181-Plat
Subcellular Origin and Tissue-Wide Synchronization of Abnormal Ca Release in the Genesis of Ca-Dependent Atrial Arrhythmia
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Introduction. Abnormal diastolic Ca release (DCR) from the sarcoplasmic reticulum has been implicated in both ventricular as well as atrial fibrillation (AF). Atrial cells lack the extensive T-tubule network that facilitates Ca signaling in ventricular myocytes. How the distinct structural organization of atrial cells affects the genesis of AF is currently unknown.
Methods and Results. We used rapid 2D confocal imaging to map Ca changes in atrial single myocytes and tissue preparations derived from CASQ2 knockout (CASQ2.KO) mice manifesting Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT). Isolated CASQ2.KO atrial cells showed frequent local and cell-wide DCR events when exposed to isoproterenol. DCR predominantly originated nearly simultaneously at several ''eager'' sites localized at cell periphery. The distribution of latencies to local DCR events in CASQ2.KO cells displayed a left-ward shift consistent with abbreviated RyR2 refractoriness associated with CPVT. This abbreviated RyR2 refractoriness translated into highly synchronous spontaneous Ca oscillations across the intact atrial tissue. Conditional overexpression of SERCA2a in CASQ.KO mice exacerbated proarrhythmic Ca oscillations by increasing the proportion of interior eager release sites and enhancing DCR synchronicity in both isolated atrial cells and atrial tissue preparations.
Conclusions. Our results suggest that aberrant DCR involves a certain set of eager Ca release sites that 1) only in part coincide with sites involved in normal EC coupling at cell periphery and 2) act as "pacemakers" for spontaneous Ca oscillations present in atrial cells. Due to abbreviated release site refractoriness, DCR is synchronized between neighboring cells thereby leading to tissue-wide Ca oscillations that may form basis for AF. SERCA2a overexpression exacerbates arrhythmic Ca oscillations by further enhancing DCR synchronicity.

2182-Plat
Hyperphosphorylation of RyRs Underlies Triggered Activity in Transgenic Rabbit Model of LQT2 Syndrome
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Loss-of function mutations in HERG potassium channels are associated with ventricular tachycardia and sudden cardiac death caused by stress. We aimed to investigate changes in Ca homeostasis in cardiomyocytes derived from LQT2 hearts and to determine whether these changes contribute to

arrhythmogenic early afterdepolarizations (EADs) characteristic of LQT2 myocytes under β-adrenergic stimulation. Parameters of Ca handling were measured in ventricular myocytes isolated from normal and LQT2 hearts using confocal Ca imaging and whole-cell patch clamp. Ca imaging revealed no LQT2-mediated changes in amplitude of Ca transients and SR Ca content under basal conditions. Experiments in saponin-permeabilized cells using SR-entrapped low-affinity Ca indicator revealed enhanced RyR-mediated SR Ca leak and SERCA-mediated Ca uptake in LQT2 myocytes. Correspondingly, western blot analyses using phospho-specific antibodies showed that phosphorylation of both RyR and phospholamban is significantly higher in LQT2 CMs vs. controls at both PKA and CaMKII sites. In the presence of isoproterenol (50 nM) LQT2 CMs exhibited diminished Ca transient amplitudes and SR Ca content compared to controls. Stimulation of LQT2 CMs with isoproterenol resulted in prolongation of plateau of action potentials accompanied by aberrant Ca releases and phase 3 EADs, in contrast to controls. Importantly, preincubation of LQT2 CMs with CaMKII inhibitor KN93 (10 min, 500 nM) prevented ISO-mediated changes in AP duration, disturbances in Ca handling and EADs. Analysis of CaMKII activity revealed no differences between LQT2 and control heart tissues, while Western-blot analysis demonstrated ~30% decrease in the abundance of catalytic subunit of protein phosphatase type 1 (PP1). These data suggest that hyperactive RyRs due to adaptive reduction in PP1 activity and thereby RyR hyperphosphorylation is a key contributor to enhanced triggered activity in hereditary LQT2 syndrome.

2183-Plat
Rapid Calcium Modulation in Cells: Direct Intracellular Access using Nanostraws
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Intracellular calcium plays a role in a host of cellular functions ranging from synaptic transmission to gene expression. As a transient, short-range signaling molecule, calcium is often presented in complex signal patterns, including oscillations and bursts. The concerted action of pumps, channels, and buffers in conjunction with the ion-impermeable cell membrane tightly regulates these calcium signals spatially and temporally but simultaneously impedes our ability to study the action of calcium. Many techniques measure changes in intracellular calcium, providing insights into the specificity of these signal patterns, but fewer are capable of controlling calcium levels in cells. Patch clamp pipette access can be used to modulate calcium in small numbers of cells, but larger scale control often requires disruption of normal calcium signaling using drugs such as thapsigargin, in order to couple intracellular calcium to an external calcium signal. Here we present a new technology to rapidly modulate intracellular calcium. Using supported nanotubes called ''nanostraws,'' we demonstrate direct ionic delivery into cells. The dimensions of the nanostraws (~100 nm x 1 μm tall) allow them to spontaneously penetrate the cell, thereby allowing calcium to bypass the lipid membrane and its constituent calcium channels through the nanostraws. Calcium delivery is controlled by fluid flow, and intracellular calcium oscillations can be induced and modulated in amplitude and frequency. While previous studies have perturbed long-term cell behavior using nanostraws and similar systems, here we apply the technique to transient signaling by rapid intracellular calcium modulation and characterize the spatiotemporal delivery using calcium indicators (e.g. Fluo-4, GCaMP6). By circumventing the intrinsic calcium regulatory mechanisms, nanostraws provide direct access and the ability to mimic biological calcium oscillations, adding a new method for decoding the role of calcium signal patterns and their effects on downstream signaling.

Platform: Ion Channels and Disease

2184-Plat
A Robust High-Throughput Assay for Thermodynamic Correctors of the Predominant Molecular Defect Causing Cystic Fibrosis
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Cystic fibrosis (CF) is a chronic lethal genetic disease, which is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. F508del - deletion of a single phenylalanine residue at position 508 - is the most common lethal mutation found in CF patients. Fully synthesized F508del-CFTR is rapidly degraded in the endoplasmic reticulum due to F508del-induced misfolding, preventing proper transport of the protein to the epithelial cell membrane. In previous work, we have demonstrated that F508del facilitates partial unfolding and consequent aggregation of the first nucleotide-binding domain in human CFTR (hNBD1). This F508del-faciliated aggregation is likely a key contributor in F508del-CF pathogenesis. These findings established a foundation to develop a new high-throughput biophysical screen to identify compounds that directly bind to the hNBD1 domain to prevent the F508del-hNBD1 aggregation *in vitro* and thereby stabilize F508del-CFTR *in vivo*. We used computational protein engineering to construct an equivalently stable hNBD1 variant that reacts with a visible fluorophore exclusively at a single labeling site. Fluorescence self-quenching of this labeled domain upon unfolding/aggregation provides a robust assay for stabilizing compounds, and this assay gives a Z-factor 0.8 in high-throughput screening. We will describe thermodynamic and structural studies on compounds identified using this screen as well as progress in implementing an equivalent screen in models of full-length human CFTR. Our results demonstrate that thermodynamic stabilization of a protein via interaction of a small molecule at a site remote from a mutation can indirectly correct a genetic defect.

2185-Plat
Converting a Stimulatory ATP Binding Site to an Inhibitory One by the Disease-Associated Mutation, G551D
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G551D, the third most common pathogenic mutation in CF, exhibits an extremely low Po because ATP fails to increase its activity. Although VX-770 (Ivacaftor) improves the symptoms of patients carrying the G551D mutation, it is insufficient to completely rectify this gating abnormality. In excised inside-out patches, we found that VX-770 increases the ATP-independent Po of WT-CFTR by ~ 9-fold, a magnitude very similar to the effect of VX-770 on G551D-CFTR (12.8 ± 1.9 fold, n = 12), suggesting a common mechanism of action: VX-770 simply shifts the equilibrium of ATP-independent gating towards the open state. However, upon ATP removal VX-770-potentiated macroscopic G551D-CFTR currents increases initially by ~2-fold (1.65 ± 0.48, n = 3), followed by a slow decay. Such a biphasic time course suggests two ATP binding sites with opposite effects and different affinities. We hypothesize that the G551D mutation converts the low-affinity ATP-binding site (i.e., site 2) to an inhibitory site, whereas the high-affinity site 1 remains stimulatory. This hypothesis predicts that lowering [ATP] could actually increase the G551D-CFTR current. Indeed, replacing 2 mM ATP with 20 μM ATP increases the current by ~ 2-fold. To further test this idea, we mutated W401 and Y1219, two aromatic amino acids known to interact with ATP in site 1 and site 2 respectively. While ATP removal increases G551D/W401G currents by ~ 2-fold (2.12 ± 0.12, n = 8), a mono-phasic current decrease is seen with G551D/Y1219G-CFTR. However, the relaxation time constants for the current decay phase for G551D and G551D/Y1219 (41.8 ± 1.0 s and 32.4 ± 1.6 s respectively) are significantly longer than that of G551D/W410G (28.8 ± 0.5 s). These results point to a unique mechanism underlying CF pathogenesis by the G551D mutation.

2186-Plat
Influenza a Blockers with Reduced Resistance Formation
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Influenza A develops amantadine resistance within days in tissue culture or infection. The amantadine-resistant mutant, M2 S31N, has become globally dominant in human isolates. Infection of MDCK cells by a pandemic 2009 H1N1 strain, bearing M2 S31N, was blocked by low-micromolar concentrations for a set of amantadine analogs previously shown to block several M2-WT influenza strains from H1N1, H2N2, and H3N2 subtypes. Three sets of variants were synthesized to explore SAR properties. One demonstrated that opening the